On the pH Dependence of Class-1 RF-Dependent Termination of mRNA Translation

Gabriele Indrisiunaite [1], Michael Y. Pavlov [1], Valérie Heurgué-Hamard [2] and Måns Ehrenberg [1]

*1 - **Department of Cell and Molecular Biology,** Uppsala University, Biomedicinskt Centrum, Box 596, 75124 Uppsala, Sweden*
*2 - **Centre National de la Recherche Scientifique,** FRE3630, University Paris Diderot Sorbonne Paris Cité Institut de Biologie Physico-Chimique, 13 Rue Pierre et Marie Curie, 75005 Paris, France*

***Correspondence to Måns Ehrenberg:** ehrenberg@xray.bmc.uu.se.*
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Abstract

We have studied the pH dependence of the rate of termination of bacterial protein synthesis catalyzed by a class-1 release factor (RF1 or RF2). We used a classical quench-flow technique and a newly developed stopped-flow technique that relies on the use of fluorescently labeled peptides. We found the termination rate to increase with increasing pH and, eventually, to saturate at about 70 s^{-1} with an apparent pK_a value of about 7.6. From our data, we suggest that class-1 RF termination is rate limited by the chemistry of ester bond hydrolysis at low pH and by a stop-codon-dependent and pH-independent conformational change of RFs at high pH. We propose that RF-dependent termination depends on the participation of a hydroxide ion rather than a water molecule in the hydrolysis of the ester bond between the P-site tRNA and its peptide chain. We provide a simple explanation for why the rate of termination saturated at high pH in our experiments but not in those of others.

Introduction

Bacterial class-1 release factors RF1 and RF2 terminate ribosomal protein synthesis at A-site codons UAA or UAG and UAA or UGA, respectively [1]. Each release factor (RF) induces hydrolysis of the ester bond between peptide and P-site-bound tRNA, which allows for release of the finished peptide chain. Termination is completed by rapid dissociation of RF1 or RF2 promoted by the class-2 release factor RF3 in a GTP-hydrolysis-dependent manner [2–4] and followed by ribosomal recycling back to a new round of initiation [5], as reviewed in Refs. [6] and [7]. In eukaryotes, a single class-1 release factor (eRF1) recognizes all three stop codons [8], while the function of the class-2 release factor eRF3 has remained obscure [9–11]. Prokaryotic release factors RF1 and RF2 have similar four-domain structures with a rigid core formed by their domains 2 and 4 [7]. Interaction between RF3 and domain 1 of RF induces RF dissociation from the ribosome [12]. Domain 2 of RF interacts with the stop codon in the ribosomal decoding center (DC) on the ribosomal 30S subunit. The tip of the helical domain 3 of RF contains a Gly-Gly-Gln (GGQ) motif, which is universally conserved among all class-1 release factors of the three kingdoms [13]. This motif reaches into the peptidyl transfer center (PTC) on the ribosomal 50S subunit [14–19] and plays a major role in the induction of hydrolysis of the ester bond between the peptide and the P-site tRNA [20]. Lethal mutations have been observed for each one of the amino acid residues of the GGQ motif [21]. Substitution of either Gly residue in GGQ of RF greatly reduces RF termination efficiency [4,21,22]. The Gln residue of GGQ has been suggested to precisely coordinate a water molecule for nucleophilic attack on the ester bond of the peptidyl-tRNA in the P site [20]. Based on the pronounced flexibility of the Gly-Gly backbone, the greatly reduced RF activity by Gly substitutions has been explained as due to a fatal failure of the Gly-mutated factors to properly coordinate the attacking nucleophile [17,23]. The amide of the Q in the GGQ motifs of class-1 RFs is methylated both in prokaryotes [24,25] and in eukaryotes [26–28], suggesting an

important role of this modification for the catalytic activity of the factors.

Despite extensive biochemical experiments, structural data from crystallography and cryo-electron microscopy in conjunction with modeling approaches, crucial aspects of the mechanism of termination induced by class-1 release factors have remained obscure. One example concerns the strong pH dependence of the rate of the RF-induced peptide release from the terminating ribosome [29,30]. This rate increases almost linearly with hydroxide ion concentration over a wide pH interval without rate saturation at high pH [29]. In principle, this behavior could be due to the titration of a single proton on an essential catalytic group of release factor or ribosome with a pK_a value well above 9 [29]. This scenario is, however, rendered unlikely by the lack of identification of any titratable catalytic group of either RF or ribosome in the vicinity of the scissile ester bond between the peptide and tRNA. Alternatively, and more likely, the pH dependence could be explained by the hydroxide ion participating in the hydrolytic reaction either directly acting as a nucleophile or indirectly abstracting a proton from the attacking water molecule [29].

Another example is a putative conformational change in class-1 RFs upon stop codon recognition in the ribosomal A site. It was found that the distance between the codon-recognizing elements of domain 2 of the release factors and the GGQ motif is 25 Å in their crystal structure off the ribosome [31,32]. On the ribosome, in contrast, the distance between these two motifs is about 75 Å allowing a class-1 release factor to simultaneously interact with the DC in the 30S subunit and the PTC in the 50S subunit, thereby coupling stop codon recognition with hydrolysis of the ester bond in the peptidyl-tRNA [14–17]. Such a huge difference between structures of free and ribosome-bound class-1 release factors has led to suggestion that they change conformation during codon recognition in translation termination [19]. Comparison of the crystal structures of free (closed) and ribosome-bound (open) forms of RFs suggests that the putative conformational transition in RF may depend on rearrangements in a "switch loop" of RF connecting domains 3 and 4 [15]. From small-angle X-ray scattering data, we previously suggested the solution structure of *Escherichia coli* RF1 to be more open and clearly distinct from its crystal structure [33]. This, however, does not mean that the solution structure of RF1 is identical with its structure on the ribosome. Indeed, subsequent small-angle X-ray scattering studies suggested that the conformation of *Thermus thermophilus* RF2 in solution was closer to the crystal structure of unbound factor than to its structure on the ribosome [34]. In addition, the closed form of class-1 RFs is required for their interaction with the PrmC methyltransferase [35], demonstrating the functional relevance of the closed structure.

In the present work, we have used an optimized biochemical system to study the pH dependence of the rate of peptide release by class-1 RFs. In this system, where termination was much faster than observed previously, the rate of termination depended on the concentration of hydroxide ions in a hyperbolic manner and saturated at high pH values at around 70 s^{-1}. With support from this data set, we suggest that a hydroxide ion rather than a water molecule was the nucleophile in the termination reaction and that, at low pH, the reaction was rate limited by low hydroxide concentration. We further suggest that, at high pH, when the chemistry of peptide release was fast due to high hydroxide concentration, termination was rate limited by an earlier, stop-codon-dependent conformational change of the RF.

Results

pH dependence of RF1-catalyzed peptide release from the ribosome

We first used stopped-flow technique to study the effect of pH on the maximal rate (k_{cat}) of peptide release at saturating concentration of wild-type (methylated) release factor 1 (mRF1). Ester bond hydrolysis was monitored by release of a fluorescent-labeled tripeptide from a ribosomal release complex with a coumarin-labeled tripeptidyl-tRNA (Cm-Met-Phe-Phe-$tRNA^{Phe}$) in the P site and the A site was programmed with a UAA stop codon. In these experiments, a solution containing mRF1 was rapidly mixed in the stopped-flow instrument with another solution containing a release complex after which time courses of peptide release at different pH values were monitored at rate-saturating concentration of mRF1 (Fig. 1a). All reactions had a fast phase with pH-dependent rate, accounting for 70–80% of total peptide release (Fig. 1a and b) and a slow phase with pH-independent rate. The latter, which became increasingly visible at increasing rate of the fast phase (Fig. 1a), is rationalized in the section "The nature of the slow phase" of the supplementary information and will not be further considered in the main text. The stopped-flow determined rate constant of the fast phase, k_{cat}^{SF}, increased from 25 s^{-1} to 62 s^{-1} while the rate of the slow phase remained unaltered at about 2 s^{-1} as pH increased from 7.5 to 8.5 (Fig. 1b). In the studied pH range, peptide release was several orders of magnitude slower in the absence than in the presence of RF (Fig. S1). The rate of the fast-phase displayed asymptotic saturation with increasing pHas follows:

$$k_{cat}^{SF} = \frac{k_{max}^{SF}}{1 + 10^{(pK_a^{obs} - pH)}} = k_{max}^{SF} \frac{[OH^-]}{K_b^{obs} + [OH^-]} \qquad (1)$$

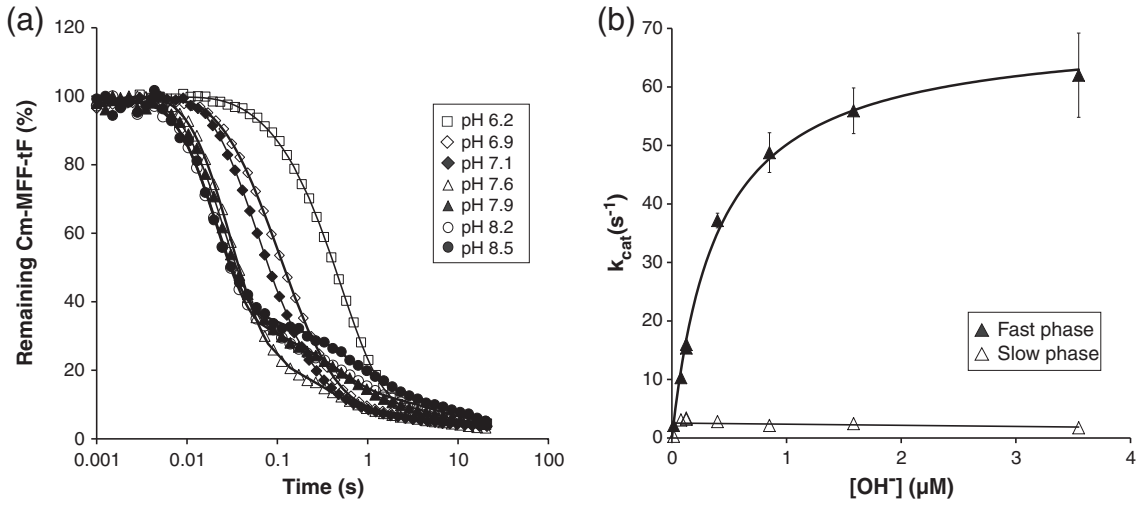

Fig. 1. The pH dependence of peptide release by methylated (wild type) RF1 measured with stopped-flow technique. Release complexes containing ribosomes with fluorescent-labeled MFF tripeptide in the P site and UAA stop codon in the A site were reacted with saturating amounts of mRF1. (a) Time courses of peptide release by mRF1 at different pH values. (b) Dependence of the release rates of the fast (▲) and slow (Δ) phases on OH^- concentration in the reaction mix.

Here, pK_a^{obs} corresponds to the pH value at which k_{cat}^{SF} was half-maximal. The constant K_b^{obs} in Eq. (1) corresponds to the concentration of hydroxide ion at which k_{cat}^{SF} was half-maximal. It is related to pK_a^{obs} through $K_b^{obs} = 10^{(pK_a^{obs}-14)}$. The parameters k_{max}^{SF} and pK_a^{obs} in Eq. (1) were estimated as 70 s^{-1} and 7.60, respectively (Table 1). Implicit in Eq. (1) is that the interpretation of the pH dependence [36] is rendered ambiguous by the multistep character of the termination reaction and the unknown mechanism of ester bond hydrolysis. In general, termination involves RF binding to a pretermination ribosomal complex R_{pre} with association rate constant k_a[RF], a putative conformational change of RF with rate constant k_{conf} [19], ester bond hydrolysis with a pH-dependent rate constant k_{hydr} and peptide release from the postterminated ribosome R_3 with rate constant k_{diss} (Fig. 2). The pH dependence for the rate of termination may arise through inhibition by proton titration of a catalytically essential group [37]:

$$k_{hydr} = \frac{k_c}{1 + [H_3O^+]/K_a} = \frac{k_c}{1 + 10^{pK_a - pH}}, \quad (2)$$

where $pK_a = -\log_{10}K_a$. Alternatively, the pH dependence may have arisen due to a hydroxide ion acting directly as a nucleophile or by abstracting a proton from the attacking water molecule [29]. In this case:

$$k_{hydr} = \frac{k_c}{1 + K_b/[OH^-]} = \frac{k_c}{1 + 10^{pK_a - pH}} \quad (3)$$

Here, k_c is the rate of the reaction when the active center is saturated with hydroxide ion and $pK_a = 10^{K_b - 14}$. Comparison of Eqs. (2) and (3) shows that the two alternative mechanisms are kinetically indistinguishable.

At saturating concentration of RF, the time of factor binding to the ribosome, $1/(k_a[RF])$, is negligible. Then, the time, $1/k_{cat}^{SF}$, is given by (Fig. 2):

$$1/k_{cat}^{SF} = 1/k_{conf} + 1/k_c(1 + 10^{pK_a - pH}) + 1/k_{diss} = 1/k_{max}^{SF} + (1/k_c)10^{pK_a - pH} \quad (4)$$

Experimental fulfillment of the saturation condition was validated by the lack of response of the rate of the fast phase to varying RF concentration (see Table S1).

It follows that pK_a^{obs} in Eq. (1) is given by [36]:

$$pK_a^{obs} = pK_a + \log_{10}(k_{max}/k_c) \quad (5)$$

It also follows that when $k_{max} \approx k_c$ then $pK_a^{obs} \approx pK_a$. When, in contrast, $k_{max}^{SF} \ll k_c$, then pK_a^{obs} is greatly down shifted in relation to pK_a.

Table 1. Saturated rates k_{max} and pK_a values of release reaction

RF	Peptide	k_{max}	pK_a^{obs}	K_b^{obs} (μM)
mRF1	Cm-MFF	70.0 ± 1.6	7.60	0.39 ± 0.03
mRF1	fMFF	57.1 ± 11.6	8.23	0.71 ± 0.38
uRF1	Cm-MFF	61.9 ± 3.7	8.48	3.05 ± 0.28
uRF1	Mq-MFF	62.9 ± 3.5	8.11	1.28 ± 0.17
uRF1	fMFF	57.5 ± 1.9	7.76	1.73 ± 0.15
mRF2	Mq-MFF	47.4 ± 1.9	7.46	0.29 ± 0.04
uRF2	Cm-MFF	45.3 ± 2.4	8.00	1.00 ± 0.1
uRF2	Mq-MFF	48.5 ± 1.4	7.86	0.72 ± 0.04

pK_a^{obs} corresponds to the pH value and K_b corresponds to the OH^- ion concentration at which the peptide release proceeds with the rate equal to half of k_{max}. The K_b values are related to the pK_a^{obs} values according to $K_b^{obs} = 10^{(pK_a^{obs}-8)}$.

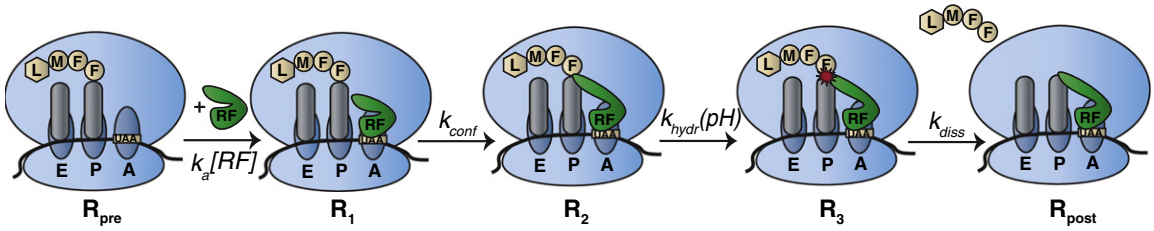

Fig. 2. The sequence of events upon mixing release factors (RF) with the release complexes. Firstly, the RF binds to a pretermination ribosomal complex R_{pre} with association rate constant k_a[RF]. Then, RF undergoes a putative conformational change with the rate constant k_{conf} followed by ester bond hydrolysis with the pH-dependent rate constant k_{hydr}. The hydrolyzed peptide is released from the postterminated ribosome R_3 with the rate constant k_{diss}. The label (L) is radioactive ^{3}H in quench-flow experiments or fluorescent coumarin derivative in stopped-flow experiments.

pH dependence of RF1-catalyzed ester bond hydrolysis

We note that, in the stopped-flow experiments described in the previous section, the main fluorescent change occurred upon peptide release from the ribosome and not at the step of ester bond hydrolysis between peptide and tRNA (Fig. 2). To assess the influence of the rate of peptide chain dissociation, k_{diss}, after ester bond hydrolysis on the maximal rate of termination, k_{cat}, we performed quench-flow experiments in which radioactively labeled peptide was released by mRF1 from tripeptidyl-tRNA in P site of the ribosome containing UAA codon in the A site. These quench-flow experiments monitored the rate of ester bond hydrolysis, k_{cat}^{QF}, preceding fMFF peptide dissociation from the ribosome:

$$1/k_{cat}^{QF} = 1/k_{conf} + 1/k_c\left(1 + 10^{pK_a - pH}\right) = 1/k_{max}^{QF} + (1/k_c)10^{pK_a - pH} \quad (6)$$

The biphasic kinetics of this reaction in the whole pH range (Fig. 3) was similar to that measured with stopped-flow technique and fluorescent-labeled peptidyl-tRNA (Figs. 1a and 3a). The fast-phase rate increased with pH and saturated at high pH (Figs. 1b and 3b). This implies that the rate-limiting termination step at high pH was not k_{diss} but either the catalytic rate k_c or a conformational change in RF1 upon A-site binding, k_{conf} (see Fig. 2). To distinguish between these two cases, we extended the stopped-flow experiments by including methylated and unmethylated variants of both RF1 and RF2 and yet another fluorescence label on the tripeptidyl-tRNA in the P site, as described in the next section.

Effects of methylation deficiency and tripeptide labeling on termination kinetics

The GGQ motif of class-1 RFs directly interacts with the PTC of the 50S ribosomal subunit [14–19,23]. Methylation deficiency in the GGQ motif was therefore

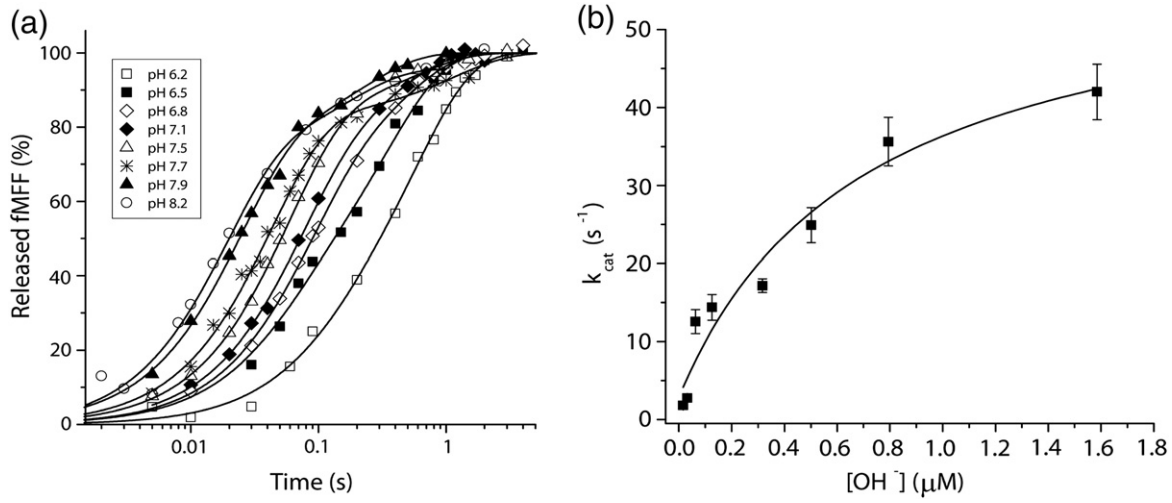

Fig. 3. The pH dependence of [^{3}H]fMFF peptide release by methylated (wild type) RF1 measured with quench-flow technique. (a) Time courses of peptide release by mRF1 at different pH values. (b) Dependence of the release rate on OH^- concentration in the reaction mix.

expected to affect the chemistry of the termination reaction, that is, k_c and pK_a in Eq. (2), but not a preceding step, k_{conf} (Fig. 2), in which the RF accommodated into the A site of the ribosome. Similarly, any effect on the termination kinetics by differently labeled tripeptides on the P-site tRNA would primarily be expected to affect k_c and pK_a rather than k_{conf}. Following this lead, we studied the pH dependence of termination with an unmethylated form of RF1 (uRF1) and differently labeled tripeptides. We used this form of RF1 both in stopped-flow experiments in combination with ribosomal release complex containing Cm-Met-Phe-Phe-tRNA^{Phe} or the differently labeled Mq-Met-Phe-Phe-tRNA^{Phe} (Fig. 4; see Materials and Methods for details on the Mq label) and in quench-flow experiments in combination with tritium-labeled fMet-Phe-Phe-tRNA^{Phe} (Fig. 5). We also used stopped flow to study the pH dependence of termination with methylated RF2 (mRF2) in combination with Mq-labeled tripeptide and unmethylated RF2 (uRF2) in combination with Mq- and Cm-labeled tripeptide on the P-site tRNA (see Figs. S2 and S3). The results of all these experiments are summarized in terms of k_{max} and pK_a^{obs} values in Table 1.

It is seen that the k_{max} values varied little between the combinations of RF and tripeptide variants: all k_{max} values were in the range 57 s^{-1} to 70 s^{-1} in the RF1 and about 47 s^{-1} in the RF2 involving cases. This small variation in k_{max} contrasts the large differences in pK_a values between the different variants, which is expressed as large k_{cat} value differences at lower pH values, as illustrated in Table 2 for pH equal to 7.5. For instance, methylation deficiency in RF1 terminating with Cm-MFF-tRNA^{Phe} in the P site reduced the pH-saturated rate of termination, k_{max}, by only 10% from 70 s^{-1} to 62 s^{-1} but it reduced k_{cat} at pH 7.5 as

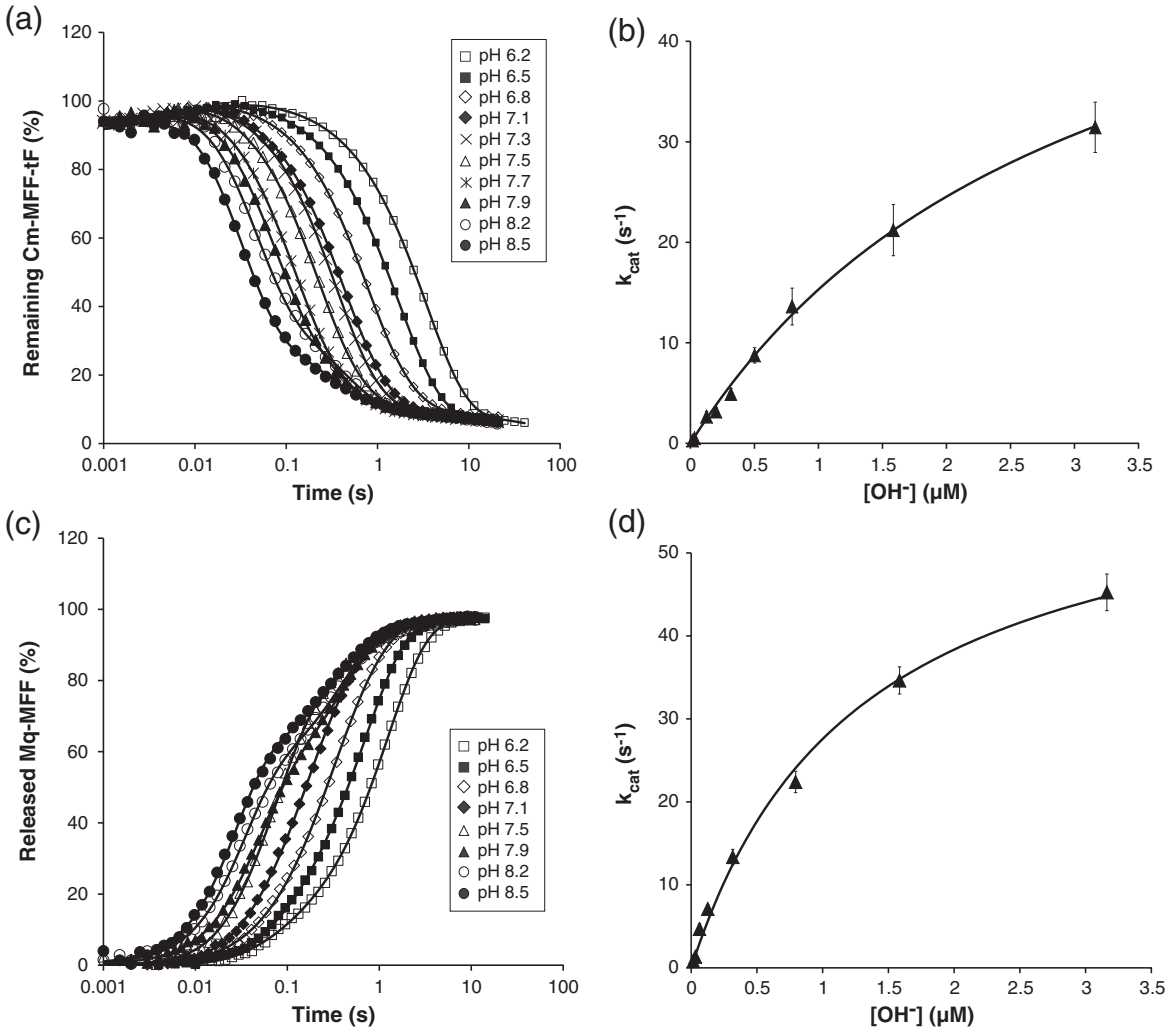

Fig. 4. The pH dependence of the release of Cm-MFF and Mq-MFF tripeptides by unmethylated RF1 (uRF1). Time courses of peptide release are shown in (a) for Cm-MFF and in (c) for Mq-MFF tripeptide. The dependences of the rate of fast phase of release on OH^- concentration are shown in (b) for Cm-MFF and in (d) for Mq-MFF tripeptide.

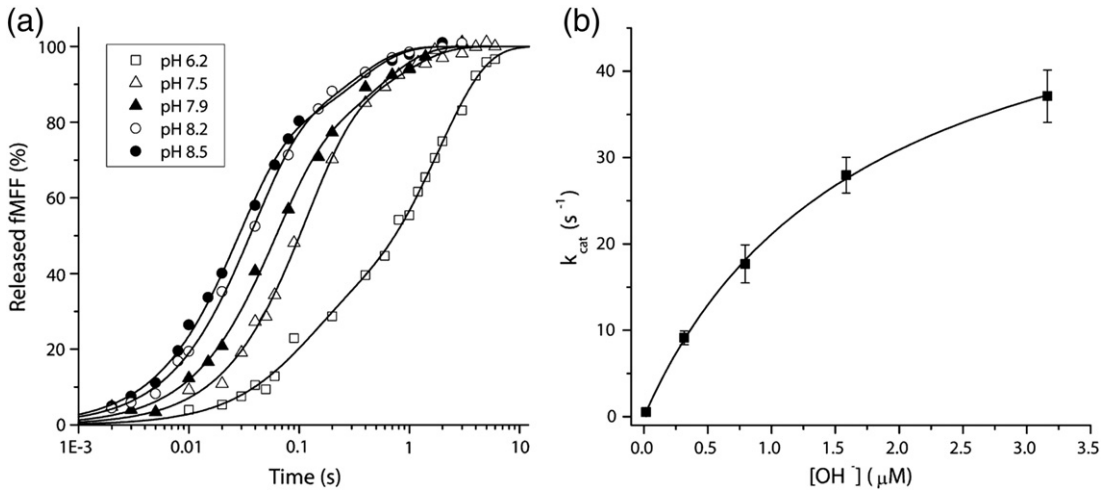

Fig. 5. The pH dependence of [^{3}H]fMFF peptide release by unmethylated RF1 (uRF1) measured with quench-flow technique. (a) Time courses of peptide release at different pH values. (b) Dependence of the release rates on OH^- concentration in the reaction mix.

much as 5-fold from 31 s^{-1} to 6 s^{-1}. These results strongly suggest that the k_{max} values originate in a rate-limiting step preceding the chemistry of termination, which we tentatively ascribe to a conformational change in the class-1 RFs upon entry into the A site as previously suggested [19]. In contrast, variations in k_{cat} at pH 7.5 reflected variations in the rate, k_{hydr}, of the chemical step due to variability in k_c for different substrates in the reaction center [Eq. (4)]. The differences in k_{cat} at pH 7.5 were also pronounced for the release of fM peptide mimic by methylated RF1 and RF2 from the ribosomal complexes containing fMet-tRNA in the P-site and a UAA stop codon in the A site (see Table 3 and Fig. S5). Interestingly, fM was released by mRF2 much more slowly (8.2 s^{-1}) than fMFF tripeptide (22.6 s^{-1}) (Tables 2 and 3). The use of the tripeptide fMFF in our pH titration experiments instead of an fM peptide mimic may offer at least a partial explanation for the much faster rate of release observed here than that in Ref. [29] (see Discussion).

Aminolysis and peptide release at high pH

The polymix buffer used in our experiments [38] contained high concentrations of the alkyl-amines spermidine and putrescine. At high pH, their amino groups would have been deprotonated and could have acted as nucleophiles competing with hydrolytic reaction by hydroxide ions or water [39]. To decide if aminolysis played a significant role in the chemistry of class-1 RF-induced termination, we used HPLC to analyze the products of termination reaction as performed at pH 7.3 or 8.5. The HPLC profiles (see Fig. S6) did not indicate the presence of aminolysis products: the total counts in the HPLC peaks were almost identical at pH 7.3 and 8.5 and there were no additional peaks that could have contained aminolysis products. The absence of detectable aminolysis even at high pH values is in line with the previous conclusion that RFs specifically exclude nucleophiles larger than water from the PTC of the ribosome [22].

Discussion

Saturating rate of termination at high pH

In line with previous observations, we have found that the class-1 RF-dependent rate of termination of ribosomes with peptidyl-tRNA in the P site increases with increasing pH [29,30]. We have also found that

Table 2. Release rates of differently labeled MFF tripeptide by methylated and unmethylated RFs at pH 7.5. A_{fast} (%) is the percentage of the fast phase of peptide release from the total amplitude.

	Stopped flow				Quench flow	
	Mq-MFF		Cm-MFF		fMFF	
RF	k_{cat} (s^{-1})	A_{fast} (%)	k_{cat} (s^{-1})	A_{fast} (%)	k_{cat} (s^{-1})	A_{fast} (%)
mRF1	26.7 ± 0.3	82 ± 7	30.6 ± 0.3	82 ± 7	17.2 ± 0.9	79 ± 7
uRF1	15.0 ± 0.2	71 ± 12	5.9 ± 0.1	74 ± 8	9.1 ± 0.8	79 ± 7
mRF2	25.7 ± 0.4	81 ± 7	26.2 ± 0.3	86 ± 4	22.6 ± 1.8	89 ± 4
uRF2	15.9 ± 0.2	79 ± 9	11.2 ± 0.2	73 ± 6	11.2 ± 0.7	82 ± 8

Table 3. Release rates of differently labeled Met by methylated RFs at pH 7.5. A_{fast} (%) is the percentage of the fast phase of peptide release from the total amplitude.

	Stopped flow				Quench flow	
	Mq-Met		Cm-Met		fMet	
RF	k_{cat} (s^{-1})	A_{fast} (%)	k_{cat} (s^{-1})	A_{fast} (%)	k_{cat} (s^{-1})	A_{fast} (%)
mRF1	—	—	—	—	12.7 ± 0.3	69 ± 7
mRF2	14.4 ± 0.2	85 ± 1	2.76 ± 0.03	75 ± 1	8.2 ± 0.9	80 ± 12

the termination rate saturates at an upper limit, k_{max}, with an apparent pK_a value, pK_a^{obs}, well below 9. In experiments performed at 37 °C with wild-type RF1 and tripeptidyl-tRNA in the P site, we estimated k_{max} and pK_a^{obs} as 70 s^{-1} and 7.6, respectively (Table 1). In principle, this could mean that there exists a previously unknown, catalytically essential group in RFs or on the ribosome with an authentic pK_a value of 7.6. To date, however, no candidate for such a group has been identified in ribosomal termination complexes [17,20,29,30,40] and previous experiments have invariably failed to detect significant saturation of the rate of termination at pH values up to 9 [29,30]. Why, then, do we observe early saturation of the pH-dependent rate of termination while other groups do not?

To provide tentative answers to this question, we first note that there is a huge difference between the estimated rate of termination in the present and in previous studies [4,24,29,30,41]. For instance, Kuhlenkoetter *et al.* estimated an RF2-dependent rate of termination as about 1 s^{-1} at 37 °C and pH 7.5 with fMet-tRNA^{fMet} in the ribosomal P site [29]. Here, in contrast, we estimate the RF2-dependent rate of termination with tripeptidyl-tRNA in the P site and at the same temperature and pH as 23 s^{-1} (Table 2). One reason for this remarkable rate difference may be that termination is faster for peptidyl-tRNAs with peptides longer than dipeptides due to, presumably, more optimal positioning of the peptidyl-tRNA in the PTC [24]. Another reason may be the choice of buffer system: when our optimized polymix buffer [38] was replaced with Tris/Bis-tris buffer system as in Ref. [29], the rate of termination with fluorescent-labeled initiator Mq-Met-tRNA^{fMet} in P site decreased from about 20 to 5 s^{-1} (Fig. S8). Whatever the reasons are, the fact that termination is much faster in our system than in those of others not only suggests that our biochemistry is closer to that of living cells but also provides a key to explain the saturation of the release rate at high pH that we observe.

That is, since at low pH we observe an almost linear increase in the rate of termination with hydroxide ion concentration, the chemistry of ester bond hydrolysis is rate limiting for termination at low pH also in our system. Then, as the hydrolytic rate constant increases with pH, the other steps, leading from RF association to the A site via ester bond hydrolysis to dissociation of peptide from the ribosome (Fig. 2), will become rate limiting. However, this will occur at a much lower pH in our assay system than in those of others. That is, in the latter systems, the rate constant for the chemistry of termination may, in spite of its pH sensitivity, remain rate limiting over the whole experimentally accessible pH range. In this scenario, the pK_a^{obs} values reported here (Table 1) are so-called kinetic pK_a values [42] greatly down shifted in relation to the authentic pK_a value of the chemical step by a slow, pH-independent step on the reaction pathway [42,36]. What, then, could be the rate-limiting steps at high pH?

One candidate is the step following ester bond hydrolysis in which the peptide chain dissociates from the ribosome. This step, with the rate constant k_{diss} (Fig. 2), contributes to the overall termination time as measured by stopped flow, with fluorescence detection, but not by quench flow, monitoring all steps up to but not further than the hydrolytic reaction. Since, however, the overall termination rates determined by stopped-flow and quench-flow techniques vary similarly with pH (Table 1), dissociation of released peptide cannot be rate limiting at high pH.

Another candidate is the step in which RF binds to the ribosomal A site. Its rate is k_a[RF] (Fig. 2) and varies in proportion to the RF concentration. This step cannot, however, be rate limiting at high pH since the overall rate of termination varies insignificantly with RF concentration at all pH values (Table S1). This result is fully in line with previous estimates of the rate constant for RF1 association to the UAA-programmed pretermination ribosome [43]. These estimates suggest that the association time at 4 μM RF concentration at 37 °C is significantly smaller than 7 ms and is thus not rate limiting at high pH.

The positive exclusion of the A-site binding step and the peptide dissociation step from the ribosome as candidates for a rate-limiting step at high pH suggests a step after RF binding to the ribosome but before ester bond hydrolysis to be the most plausible candidate for rate limitation at high pH: the step with rate constant k_{conf} in Fig. 2.

This could be a large conformational change of a RF, as suggested by Rawat *et al.* [19] and others [14,15], or of the ribosome, as proposed by Green and coworkers [44,45].

From a comparison of free and ribosome-bound RF1, Laurberg *et al.* [15] proposed how a large-scale conformational change of the factor from its closed [32] to its open [16,46] form could be induced by stop codon

recognition. In this model, a rearrangement of residues 286–293 in the switch loop region [15], initiated by stop codon recognition, directs the tip of domain 3 of the RF, containing the GGQ motif, into the PTC. Thereby, the switch loop itself is packed into a pocket formed between the 23S rRNA and the RF [15]. Korostelev *et al* proposed a similar rearrangement for RF2 [14]. It should be noted, however, that small-angle X-ray data are compatible with an open solution structure of RF1 [33], similar to the ribosome-bound factor [16,46] and distinct from the crystal structure of RF1 alone [32]. This could mean that the putative conformational change of the factor upon stop codon recognition is less dramatic than was originally proposed [19].

Green and coworkers suggested that the DC has different conformations depending on whether an A-site-bound RF interacts with a cognate stop or a near-cognate sense codon [44]. The same group also performed experiments with Fe(II)-derivatized RFs and demonstrated that hydroxyl radicals in position 292 of the switch loop of a RF cleaved the surrounding rRNA more efficiently in the presence than in the absence of a cognate stop codon [45]. These results suggested that stop codon recognition in the DC could cause the 23S rRNA regions surrounding the release factor to move closer to the RF and stabilize its active conformation [45]. Such movements of the 23S rRNA regions may occur in parallel with a conformational change of the RF itself.

The existence of a conformational change in RFs or the ribosome upon cognate codon recognition is attractive in that it rationalizes the partitioning of sense codon discrimination into a binding part and a catalytic part [41]. That is, RFs bind with a thousand-fold lower affinity to sense than to stop-codon-programmed ribosomes [41,43]. The forward rate constant leading to ester bond hydrolysis can be up to a thousand times slower for a sense than a cognate stop codon [41]. Hence, it is conceivable that the forward rate corresponds to a codon-dependent factor/ribosome activation followed by ester bond hydrolysis in analogy with activation of ternary complex for rapid GTP hydrolysis in initial codon recognition by tRNAs [47].

A striking aspect of the present data set is that the rate of termination at low pH is sensitive to modifications in the peptide of the peptidyl-tRNA and the GGQ motif of the release factors that are likely to affect positioning of the scissile bond and the attacking nucleophile in the PTC. At high pH, in contrast, the rate of termination is remarkably insensitive to these modifications: it is almost unaffected by changes in the methylation status of the GGQ motif or the presence of different fluorescent labels in the tripeptides of the peptidyl-tRNA in the P site (Table 1). Moreover, at low pH, the termination reaction responds sensitively to alterations of these variables exhibiting great changes in pK_a^{obs} value and release rate at pH 7.5 (Tables 1 and 2). This sensitivity to alterations is in line with the suggestion that the chemistry of termination is rate limiting at low pH. At the same time, the insensitivity to the alterations at high pH is fully in line with the proposal that the rate-limiting termination step corresponds to a conformational change of RF and/or ribosomal DC in response to stop codon recognition, provided that the rate of these changes is not strongly correlated with alterations in the PTC. A case to be considered is when a conformational change in a RF brings its GGQ motif into the PTC. Then, the affinity of the GGQ to the PTC may vary with its methylation status. However, the magnitude of the effect of tighter or weaker binding of the GGQ to the PTC on the termination rate will fundamentally depend on a comparison between the dissociation rate constant of GGQ from the PTC and the forward rate constant of ester bond hydrolysis. When, at high pH, the hydrolytic rate constant is much larger than the dissociation rate constant, the probability that the first GGQ binding to the PTC leads to peptide release is near 1. Accordingly, even large variations in the rate constant for dissociation of GGQ from the PTC will not significantly affect the rate of termination. Hence, conformational changes in both RF and DC remain as prime candidates for being rate limiting at high pH.

The hydroxide ion as the nucleophile in termination of protein synthesis

The reaction scheme presented in Fig. 2 assumes that the nucleophile of the hydrolytic reaction is a hydroxide ion, as previously suggested to explain the proportionality between the rate of RF2-dependent termination and hydroxide ion concentration [29]. Positioning of the hydroxide ion in the catalytic center could occur by either replacing a water molecule in that position with a hydroxide ion or an adjacent hydroxide ion abstracting a proton from a water molecule in the catalytic center (see Fig. 2 of Kuhlenkoetter *et al*. [29]). In both these scenarios, at physiological pH values, the hydrolytic rate would be proportional to the concentration of hydroxide ions in the reaction buffer. We note that the concentration of hydroxide ions is only 1 μM at pH 8. This means that if the catalytic center has the same affinity to water and hydroxide ions, the probability that it contains a hydroxide ion and not a water molecule is only 1 in 55 million. It is therefore not unlikely that the catalytic center formed by the PTC of the ribosome and the class-1 RF has evolved so as to greatly favor hydroxide ion to water molecule binding.

In contrast to these expectations, it has been proposed from crystallographic data that a water molecule and not a hydroxide ion is favored in the catalytic center of termination and that the amide oxygen of Gln in the GGQ motif of the RFs forms a

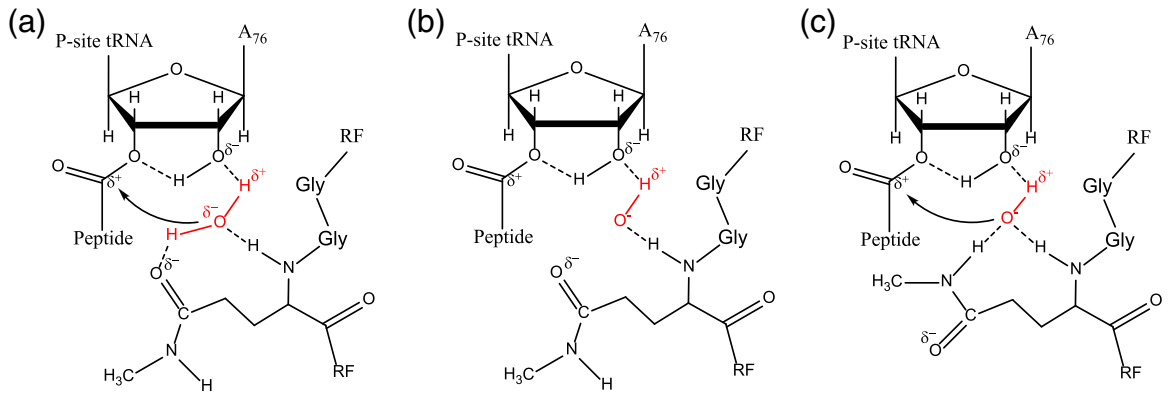

Fig. 6. Comparison of peptide release reaction schemes with water and hydroxide ion as nucleophiles. (a) Reaction scheme with water as the nucleophile accommodated as proposed by Jin *et al.* [17]. (b) An OH^- ion could not replace water in the same scheme because it would be repelled by the negatively charged carbonyl group of Gln side chain. (c) If the Gln side chain is flipped as shown here, OH^- could be accommodated and stabilized by a hydrogen bond donated by the Gln amide group.

hydrogen bond with the reactive water molecule [17] (Fig. 6a). This oxygen has a partial negative charge that would repel the negatively charged hydroxide ion (Fig. 6b) and seemingly speak against the hypothesis that the hydroxide is the nucleophile in termination of protein synthesis. However, this interpretation of structural data rests on the tacit assumption of a particular positioning of the amide group of the Gln in GGQ [17]. If this group were flipped as illustrated (Fig. 6c), a hydroxide ion would be favored in relation to a water molecule in the catalytic center. This would greatly favor donation of a hydrogen bond from the amino group of the amide to a hydroxide ion in the catalytic center and thus speed up the chemistry of termination. It has also been proposed that the nucleophile is primarily coordinated not by a side chain but by the backbone amide of the Gln [14,48]. A mutation of glutamine to proline (the only amino acid lacking the backbone amide) resulted in complete loss of peptide release activity [14] in contrast to the smaller effects of other amino acid substitutions [21,22]. Both an OH^- ion (Fig. 6c) and a water molecule (Fig. 6a) could be coordinated by the backbone amide of Gln. However, this alternative coordination is expected to favor hydroxide anion over water in the active center due to a much stronger hydrogen bond between the amid donor and anion acceptor than between an amide donor and a neutral acceptor, like water. A definitive identification of the nucleophile in the termination reaction is still beyond reach. It will probably require high-resolution structural information on the nucleophile coordination in ribosomal termination complex in a state immediately before ester bond hydrolysis.

Conclusions

We have found strong pH dependence of the rate of class-1 release-factor-dependent termination of protein synthesis. We have observed saturation of the termination rate at high pH values. We propose that the chemistry of termination is rate limiting at low pH and that a pH-independent step is rate limiting at high pH. The latter is, we suggest, a conformational change in the class-1 release factor induced by its contact with a cognate stop codon. Finally, we propose that ester bond hydrolysis is conferred by a hydroxide ion rather than a water molecule in the catalytic center and discuss this scenario in the context of previous structural data on ribosomal termination complexes.

Materials and Methods

Chemicals and buffers

Radioactive [^{3}H]methionine was purchased from GE Healthcare. The fluorescent dyes 7-diethylaminocoumarin-3-carboxylic acid, succinimidyl ester (denoted further as Cm) and 7-methoxycoumarin-3-carboxylic acid, succinimidyl ester (denoted further as Mq) were purchased from Invitrogen. Phosphoenolpyruvate (PEP), pyruvate kinase (PK), myokinase (MK), inorganic pyrophosphatase (PPi), spermidine, putrescine and nonradioactive amino acids were from Sigma-Aldrich. Ribolock RNase inhibitor was from Fermentas. Other chemicals were from Merck or Sigma-Aldrich. All experiments were carried out in polymix-like (PM) buffer [38] containing 5 mM $Mg(OAc)_2$, 95 mM KCl, 3 mM NH_4Cl, 0.5 mM $CaCl_2$, 1 mM spermidine, 8 mM putrescine, 1 mM dithioerythritol and 30 mM Hepes. The pH of the reaction mixtures was adjusted with 0.5 M KOH or 1 M HCl.

Components of the translation system

70S ribosomes (*E. coli* strain MRE600) were purified as previously described [49]. Synthetic mRNA encoding fMet-Phe-Phe-UAAU (UUC as Phe codon) with a strong Shine–Dalgarno sequence was prepared according to

Ref. [50]. Initiation factors were purified from overproducing strains according to Refs. [51] and [52]. Elongation factors, $tRNA^{Phe}$, Phe-tRNA synthetase and Met-tRNA synthetase were prepared as in Ref. [53]. f[^{3}H]Met-$tRNA^{fMet}$ was prepared as in Ref. [52] except for the purification step. It was purified on HPLC in the same way as fluorescent-labeled Met-$tRNA^{Met}$ described below.

Expression and purification of release factors (RFs)

RFs used contained a C-terminal His6 tag. It has been shown previously not to affect the RF activity in peptide release [21]. Unmethylated release factors were overexpressed in BL21 strain (for RF1) or BL21-Gold (DE3) (for RF2). RF1(His)6 protein was produced from pVH460 plasmid, a derivative of pLV1 plasmid, under the dependence of the Ptrc promoter [25]. *prfA* gene was cloned from pET11a (prfA) plasmid [24] and modified to encode a C-terminal His6 tag. RF2Ala246 (His)6 protein was produced from pET11a (RF2alaHis6) plasmid where *prfB* gene was modified to remove the frameshift site [24] and to encode a C-terminal His6 tag.

Methylated release factors were obtained by coexpression of both RFs and PrmC/HemK methyltransferase in BL21-Gold (DE3) strain. mRF1 was produced by coexpression of RF1 from pVH460 plasmid and untagged HemK from compatible plasmid pVH470. *hemK* gene was cloned in pACYCDuet-1 plasmid between NdeI and BamHI sites from pLV(hemK) [25] giving pVH470. mRF2 was produced by coexpression of RF2 from pET11a (RF2alaHis6) and untagged HemK from compatible plasmid pW(hem11) [25], a derivative of pWSK129. RF expression was performed in LB medium supplemented with appropriate antibiotics after addition of IPTG at 1 mM final concentration when the absorbance reached 0.5 at 600 nm and incubation for 3 h. Proteins were prepared by nickel affinity chromatography in buffer 1 for RF1 [20 mM Tris–HCl (pH 7.4)] and in buffer 2 for RF2 [20 mM Tris–HCl (pH 7.4) and 2.5 mM imidazole] and were eluted with buffer 1 containing 150 mM imidazole. Proteins were dialyzed overnight against 20 mM Tris–HCl (pH 7.4). The extent of Q methylation in the GGQ motif was determined by mass spectrometry as in Ref. [25]. Methylated RF1 and RF2 were over 95% methylated, and no methylation was detected in unmethylated RF2 and $<10\%$ in unmethylated RF1.

Preparation of fluorescently labeled Met-$tRNA^{fMet}$

The fluorescent dye solution was prepared by dissolving 6 mg of fluorescent Mq or Cm in 1.2 ml DMSO. Met-$tRNA^{fMet}$ was prepared in the following way. Charging mix (2 ml) contained 150 μM [^{3}H]Met, 80 μM $tRNA^{fMet}$, 0.5 U/μl Met-$tRNA^{Met}$ synthetase, 2 mM $Mg(OAc)_2$, 2 U/μl PK, 1 U/μl MK, 1 μM PPi, 0.2 U/μl Ribolock, 2 mM ATP, 20 mM PEP, 1 mM DTE and 30 mM Hepes. After 30 min of charging at 37 °C, the tRNA was extracted with phenol/chisam, precipitated with EtOH and dissolved in ddH_2O, and NaOAc was added to 0.1 M. We added 0.5 ml of the fluorescent dye solution to 0.3 ml of the [^{3}H]Met-$tRNA^{fMet}$ solution, the reaction mix was incubated for 1 h in the dark, the rest of the fluorescent dye solution (0.7 ml) was added and NaOAc concentration was adjusted to 0.1 M. The reaction was allowed to proceed overnight in the dark at 4 °C after which the modified tRNA was precipitated with ethanol, extracted twice with phenol/chisam, ethanol precipitated again and dissolved in ddH_2O.

Fluorescent [^{3}H]Met-$tRNA^{Met}$ was further purified using an HPLC system (Waters) equipped with Lichospher WP 300 RP-18 column (Merck), UV and fluorescence detectors to separate it from noncharged or nonmodified tRNA. Fluorescent [^{3}H]Met-$tRNA^{fMet}$ was eluted using a linear gradient from 16% to 36% methanol in a buffer containing 20 mM NH_4Ac (pH 5.0), 5 mM $MgCl_2$ and 400 mM NaCl. Fractions corresponding to the peak of both UV and fluorescence were tested for radioactivity by scintillation counting to confirm the presence of charged tRNA. Fractions containing most radioactivity were pooled together, concentrated in 10-kDa-cutoff centrifugal filters (Amicon) and stored in aliquots at −80 °C.

Preparation of release complexes

Release complexes (RC) contained stalled ribosomes with peptidyl-tRNA with labeled Met-Phe-Phe (MFF) tripeptide in the P site and a stop codon UAA in the A site. They were prepared in the following way. The ribosome mix was prepared in PM buffer and contained 2 μM 70S ribosomes; 3.5 μM labeled Met-$tRNA^{fMet}$; 3.5 μM mRNA; 1 mM GTP; 1 mM ATP; 2 mM PEP; 2 μM each of IF1, IF2 and IF3; and additional 2 mM $Mg(OAc)_2$. The factor mix prepared in PM buffer contained 10 μM EF-Tu, 1 μM EF-Ts, 3.4 μM EF-G, 5 μM $tRNA^{Phe}$, 0.2 mM Phe, 0.09 U/μl PheRS, 1 mM GTP, 1 mM ATP, 2 mM PEP, 0.5 mM DTE, additional 2 mM $Mg(OAc)_2$, 0.01 U/μl of each PK and MK and 0.02 U/μl Ribolock. Both mixes were preincubated for 20 min at 37 °C, reacted together for 40 s at 37 °C and cooled on ice to stop the reaction. Mg^{2+} concentration was increased by 4 mM with $Mg(OAc)_2$. RCs were purified by ultracentrifugation through a sucrose cushion containing 1.1 M sucrose in PM with 4 mM additional $Mg(OAc)_2$. We layered 500 μl of reaction mix over 500 μl of the cushion and centrifuged it in a swing-out rotor (S55S; Sorvall) at 258,000*g* for 2 h at 4 °C in a Sorvall RC M150 GX ultracentrifuge. The pellets were dissolved in PM buffer and RC concentration determined by scintillation counting. Purified RCs were stored in aliquots at −80 °C. To estimate the quality of RCs, we reacted them with saturating concentrations of RF1 for 40 s to fully release the peptide and analyzed the reaction products by HPLC. The peptide was released from about 80% of available RCs. Over 80% of the released peptide was MFF, but small amounts of MF and M were present (see Fig. S7). No release was observed in RF absence.

Peptide release experiments

Separate RC and RF mixes with adjusted pH were prepared in PM containing 0.1 mM of each Tyr and Phe. The RC mix contained 0.04 μM RCs and 0.4 U/μl Ribolock. The RF mixes contained experimentally determined saturating concentrations of release factors in at least 100-fold molar excess over RC to avoid the binding rate of the RF becoming rate limiting. All peptide release experiments were performed at 37 °C.

Stopped-flow experiments

Equal volumes of RC and RF were rapidly mixed in a stopped-flow instrument (SX-20; Applied Photophysics) and the change in fluorescence signal caused by the release of fluorescently labeled peptide was recorded as a function of time. Cm fluorescence was excited at 380 nm and measured after passing a 420-nm-cutoff filter (358 nm and 395 nm, respectively, for Mq). Each time course was averaged from 6 to 8 traces and fitted with an exponential model to obtain rate constants. Blank reactions were run to ensure that there was no change in fluorescence in the absence of RF.

Quench-flow experiments

Equal volumes of RC and RF were rapidly mixed in a quench-flow instrument (RQF-3; KinTek, Corp.), allowed to react for set amounts of time and quenched with formic acid to a final concentration of 17%. The samples were cooled on ice and centrifuged at 20,000*g* for 15 min to separate precipitated [^{3}H]MFF-tRNA from released [^{3}H]MFF peptide. The pellets were dissolved in 120 μl of 0.5 M KOH by incubation for 15 min at 37 °C. The amount of radioactivity in pellets and supernatants was determined by counting in Quicksafe Flow 2 scintillation liquid in a Beckman Coulter LS 6500 counter. Aliquots of the RC mix were taken before and after a time series to check for spontaneous hydrolysis of the peptidyl-tRNA ester linkage. They were manually quenched with formic acid to the final concentration of 17% and further treated as the rest of the samples. At pH > 8, the RC mix was cooled with ice to avoid spontaneous hydrolysis that occurs at high pH.

Data treatment

The presence of the two phases of release suggests that either the release complexes (RC) or the release factors (RF) used in our experiments were heterogeneous. In both cases, the reaction is described by the scheme:

$$A \begin{array}{l} \xrightarrow{k_1} B_1 \xrightarrow{k_3} C_1 \\ \xrightarrow{k_2} B_2 \xrightarrow{k_4} C_2 \end{array} \qquad \text{(R1)}$$

In the case of the heterogeneous RF population containing "fast" RFA and "slow" RFB release factors in a large molar excess over release complexes, one can identify A with release complexes RC so that $k_1 = k_a$[RFA] and $k_2 = k_a$[RFB], where k_a is the association rate constant (Fig. 2). The rates k_3 and k_4 are the compounded rates of the release reaction on the ribosome for the "fast" and "slow" release factor, respectively. State B corresponds to the RC complex after RF binding while state C is the state with released peptide. Fluorescent signal is then given by:

$$\text{Flu} = F_A[A] + F_B([B_1] + [B_2]) + F_C([C_1] + [C_2]) \qquad \text{(R2)}$$

Here, F_A is the fluorescence of release complex before RF binding, F_B is that after RF binding and F_C is the fluorescence after peptide release. Concentrations of different states for the reaction scheme (R1) were obtained by analytic solution of corresponding differential equation system with parameters k_1, k_2, k_3 and k_4. Experimental data were then fitted to Eq. (R2) using Marquardt algorithm to determine the rate and fluorescence parameters. The fraction of the fast phase of release, A_{fast}, was calculated as $A_{fast} = k_1/(k_1 + k_2)$.

The parameter k_3 determines the compounded rate of the fast phase of the release reaction on the ribosome.

In the case of heterogeneous ribosome population with "fast" and "slow" release complexes, the reaction scheme (R1) and its treatment is the same, except that the rate parameters k_1 and k_2 are given by $k_1 = r_f k_a$[RF] and $k_2 = (1 - r_f)k_a$[RF], where r_f is the fraction of "fast" release complexes.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jmb.2015.01.007.

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DC, decoding center; PTC, peptidyl transfer center.

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